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Physiological levels of cardiolipin acutely affect mitochondrial respiration in vascular smooth muscle cells

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ABSTRACT

Cardiolipin (CL) is a phospholipid molecule found in the inner mitochondrial membrane, where it normally associates with and activates the respiratory complexes. Following myocardial infarction, CL gets released from necrotic cells, consequently affecting neighboring tissues. We have previously demonstrated that physiological concentrations of up to 100 μM CL diminish endothelial cell migration and angiogenic sprouting. Since CL is vital to cellular life, we hypothesized that this molecule may have considerable implications on vascular smooth muscle cells bioenergetics, a key phase in atherogenesis. We examined the acute effects of physiological concentrations of CL on oxidative phosphorylation in permeabilized mice aorta using high-resolution respirometry and a substrate-inhibitor titration protocol. We found that CL significantly lowers LEAK and maximal State 3 respiration. In addition, we found that the acceptor control ratio, representing the coupling between oxidation and phosphorylation, was significantly upregulated by CL. Our findings demonstrate that *in situ* mitochondrial respiration in permeabilized smooth muscle cells is attenuated when physiological concentrations of CL are applied acutely. This could provide a novel therapy to reduce their dedifferentiation and consequently atherogenesis.

1. Introduction

Cardiolipin (CL) is a highly conserved, unique phospholipid molecule composed of four fatty acid side chains and three glycerol units found almost exclusively in the inner mitochondrial membrane (Schlame et al., 1993; Hatch, 1998; Mileykovskaya and Dowhan, 2009; Balasubramanian et al., 2015). Within these organelles, CL provides structural support and acts as a proton trap and has also been shown to promote mitophagy by serving as an external signal for damaged mitochondria (Gonzalvez and Gottlieb, 2007; Smith et al., 2011; Chu et al., 2013; Balasubramanian et al., 2015). CL is also involved in programmed cell death through anchoring of cytochrome *c* which triggers downstream apoptotic cascades (Mcmillin and Dowhan, 2002; Tuominen et al., 2002). Interestingly, CL is the only phospholipid molecule released in significant amount during cardiomyocyte perforation following a myocardial infarction (De Windt et al., 1998; Paradies et al., 1999; Lesnefsky et al., 2004) and that it is quadruple more in the human plasma than other phospholipids (Deguchi et al., 2000). CL affect neighboring cells like epithelial cells (Carnevale et al., 2015) and brain cells (Ordóñez-Gutiérrez et al., 2015). Myocardial infarctions often originate from occlusion established by the transition of normally contractile vascular smooth muscle cells (VSMCs) to a synthetic, dedifferentiated phenotype, accompanied by characteristic changes in protein composition such as a reduction of contractile filaments (Owens, 1995; Majesky, 2007; Bergdahl et al., 2013). This distinct plasticity is essential during early stages of atherogenesis, and may confer a survival advantage since it provides means for the VSMCs to respond to altered conditions in its surroundings. Although a number of transcriptional networks that facilitate VSMC dedifferentiation have been mapped, the master regulators of phenotype shift remain elusive. There are no, or very few, studies describing the significance of CL in this process. CL is vital to cellular life and it is thus natural to imagine that this substance could also play a critical role in cellular dedifferentiation. Since CL is an important phospholipid found almost exclusively in the inner mitochondrial membrane and is essential in maintaining the organelle's activities, it is likely that abnormalities in CL concentration will have significant implications for mitochondrial function and, hence, for cellular adaptations.

The primary role of mitochondria is to produce ATP through oxidative phosphorylation, conducted by complexes I–IV and the ATP

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synthase (complex V) (Duchen, 2004). This capacity relies heavily on substrate availability as mitochondria utilize molecules derived from glucose and fatty acid oxidation. A key function for the mitochondria is to maintain the proton gradient created by the passage of electrons along the complexes (Duchen, 2004; Irwin et al., 2013). Energy stored by shunting H^+ to the outer membrane fuels ATP synthesis through complex V (Schultz et al., 2001; Irwin et al., 2013). In normal conditions, CL stabilizes the respiratory complexes as well as functions as a proton trap to concentrate the proton pool and minimize the pH alterations in the mitochondrial intermembrane space (Carnevale and Bergdahl, 2015).

Despite its seemingly central role in cellular function, little is known about the effects of CL on VSMC mitochondrial respiration. The aim of this study was to investigate whether physiological concentrations of CL affect bioenergetics and mitochondrial oxygen consumption. We hypothesized that an increase in CL concentration, in ranges normally seen following cardiac ischemia, would acutely reduce the oxidative phosphorylation in VSMCs from fresh aortic tissue.

2. Materials and methods

In the experiments presented, CL at 1 and 10 μM concentrations were used. It has been reported that around 15 μg/ml (10 μM) of CL is considered physiological in human plasma (Deguchi et al., 2000).

2.1. Animals

Male C57Bl/6 mice, 7–8 months old were obtained from the Concordia University breeding colony and randomly assigned to either a control (CON) or two CL groups (1 and 10 μM, respectively). The mice were housed individually in a thermo-neutral environment (22 °C), on a 12:12 h photoperiod, provided standard dry laboratory chow as well as water ad libitum. All procedures were approved by the Animal Ethics Committee of Concordia University (#30000259) and were conducted in accordance with guidelines of the Canadian Council on Animal Care.

2.2. Experimental protocol

The animals were euthanized by CO_2 after which the aorta was removed and immediately placed in an ice-cold buffer solution (BIOPS) containing (in mM): CaK_2EGTA 2.77, K_2EGTA 7.23, Na_2ATP 5.77, $MgCl_2{\cdot}6H_2O$ 6.56, Taurine 20, Na_2 Phosphocreatine 15, Imidazole 20, Dithiothreitol 0.5, MES 50, pH 7.1. The aortic samples were gently dissected, connective tissue and fat were removed before the vessels were separated using sharp forceps in ice-cold BIOPS buffer. Two aortas were used per sample which provided approximately 6–8 mg of muscle tissue (wet weight). After dissection, the vessels were incubated in 2 mL BIOPS buffer containing 50 μg mL-1 saponin for 30 min on ice. The aortas were then washed in ice-cold buffer (MiR05) for 2 × 10 min MiR05 contains (in mM): EGTA 0.5, $MgCl_2{\cdot}6H_2O$ 3.0, K-lactobionate 60, Taurine 20, KH_2PO_4 10, HEPES 20, Sucrose 110, BSA 1g L-1, pH 7.1. Measurements of oxygen consumption were performed at 37 °C using high-resolution respirometry (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria). The respirometric measurements were done in the buffer MiR05. This technique has been described in further detail elsewhere (Kuznetsov et al., 2008; Larsen et al., 2015).

2.3. Mitochondrial respiratory measurements

To avoid potential oxygen limitation, all experiments were carried out under hyperoxygenated conditions and followed a previously described protocols (Rocha et al., 2018; Kuznetsov et al., 2008; Sahl et al., 2021). Twenty minutes prior to the addition of substrates, CL was provided to the 2 mL oxygraph chambers for a final concentration of 1 and 10 μM, respectively. Thereafter, state 2 respiration (absence of adenylates) was assessed by addition of malate (2 mM) and octanoylcarnitine (1.5 mM, ETF_L), while state 3 respiration was achieved by adding ADP (5 mM; ETF_P). This was followed by the addition of glutamate (10 mM; CI_P) and succinate (10 mM; CI + II_P), thus, achieving maximal coupled respiration with convergent electron input to complex I and II of the electron transport system. Thereafter oligomycin (2 μg mL-1) was added to block complex V,LEAK (state 4), followed by antimycin A (2.5 μM) to inhibit complex III (ROX).

2.4. Statistics

The values and ratios calculated were obtained from the stabilized plateaus following the addition of the substrates/inhibitors of the oxygen flux graphs (Fig. 1 is a representative control graph). The acceptor control ratio (ACR) was determined by dividing ADP by malate average rates of respiration. The respiratory control ratio (RCR) was calculated by dividing state 3 by state 4 average rates of respiration.

Data are presented as mean (SD) in all figures and tables. For all statistical evaluations, $P < 0.05$ was considered significant. One-way ANOVAs for the CL concentration factor were performed. Significant main effects or interactions were further analyzed by the Tukey post hoc test. The statistical analysis was performed using the software OriginPro 2015 (OriginLab Corporation, Northampton, MA, USA). Data from tissues treated with control, 1 μM and 10 μM CL were compared. Eight samples, each consisting of two randomly selected aortas were used per condition.

3. Results

To investigate whether physiological concentrations (1 and 10 μM) of CL acutely affect VSMC mitochondrial respiration, we studied the oxygen consumption rates using high-resolution respirometry.

Fig. 1 is a representative control trace of the respiratory flux from the substrate and inhibitor protocol. The red line depicts the oxygen flux in the chamber. The stabilized plateaus after each substrate/inhibitor addition were used for the following figures.

As seen in Fig. 2a, there was a significant reduction by CL in state 2 respiration after addition of the Complex I linked substrate, malate (basal, ADP-restricted) at $p < 0.05$ level for conditions [1.50 ± 0.15, 0.59 ± 0.10 and 0.43 ± 0.13 pmol/s/mg for CON, 1 and 10 μM CL, respectively] for $n = 8$ [$F(2, 21) F = 19.76$, $p = 0.00001$]. A Tukey post-hoc test revealed significant differences between CON and 1 μM ($p < 0.001$) as well as CON and 10 μM ($p < 0.001$) CL. Fig. 2b demonstrates a significant reduction of CL on lipid LEAK respiration induced by octanoylcarnitine [2.49 ± 0.10, 1.08 ± 0.21 and 0.92 ± 0.22 pmol/s/mg for CON, 1 and 10 μM CL, respectively] for $n = 8$ at the $p < 0.05$ level [$F(2, 21) F = 21.22$, $p = 0.0001$] with a Tukey post-hoc test proving significant differences between CON and 1 μM ($p < 0.001$) as well as control and 10 μM ($p < 0.0001$) CL.

Addition of ADP to stimulate state 3 respiration (Fig. 3a) demonstrated that CL significantly inhibits ADP-stimulated respiration [3.55 ± 0.18, 2.96 ± 0.36 and 2.12 ± 0.30 pmol/s/mg for CON, 1 and 10 μM CL, respectively] at the $p < 0.05$ level for $n = 8$ [$F(2, 21) F = 6.15$, $p = 0.008$]. Significant differences were detected between CON and 10 μM CL ($p < 0.01$), but not between 1 and 10 μM CL using the Tukey post-hoc test. Further stimulating Complex I respiration by addition of glutamate, but now in an ADP-stimulated environment, demonstrated an increase for CON and 1 μM CL, while the 10 μM CL did not change, when compared to the state 3 respiration (Fig. 3b) [4.17 ± 0.17, 4.46 ± 0.56 and 2.13 ± 0.34 pmol/s/mg for CON, 1 and 10 μM CL, respectively] for $n = 8$ [$F(2, 21) F = 10.48$, $p = 0.0007$]. Tukey post-hoc test revealed significant difference between control and 10 ($p < 0.01$) as well as 1 and 10 μM ($p < 0.01$) CL.

Addition of succinate (Fig. 4), a Complex II specific substrate, thus stimulating electron input to both complex I and II, revealed strong reduction by CL [11.82 ± 0.19, 7.54 ± 0.64 and 5.99 ± 0.77 pmol/s/mg for CON, 1 and 10 μM CL, respectively] for $n = 8$ [$F(2, 21) F = 26.53$, $p = 0.0001$]. A Tukey post-hoc test revealed significant differences

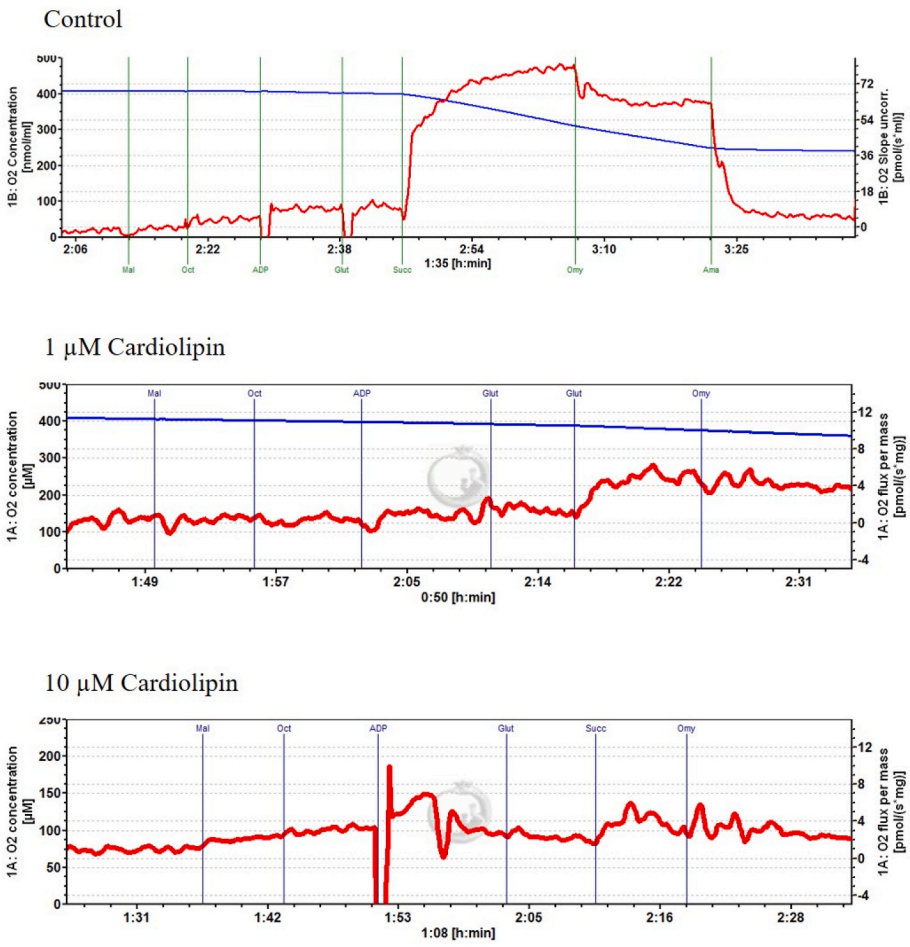

Fig. 1. Representative trace of the respiratory flux from the substrate and inhibitor protocol for the control, 1 and 10 μM CL conditions. The blue line (y-axis, left) is oxygen concentration in the chamber. Red line (y-axis, right) is oxygen flux in the chamber. Addition of substrates and inhibitors as indicated in the figure. Mal: malate; Oct: octanoyl carnitine; ADP: adenosine diphosphate; Glut: glutamate; Succ: succinate; Omy: oligomycin; Ama: antimycin A. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

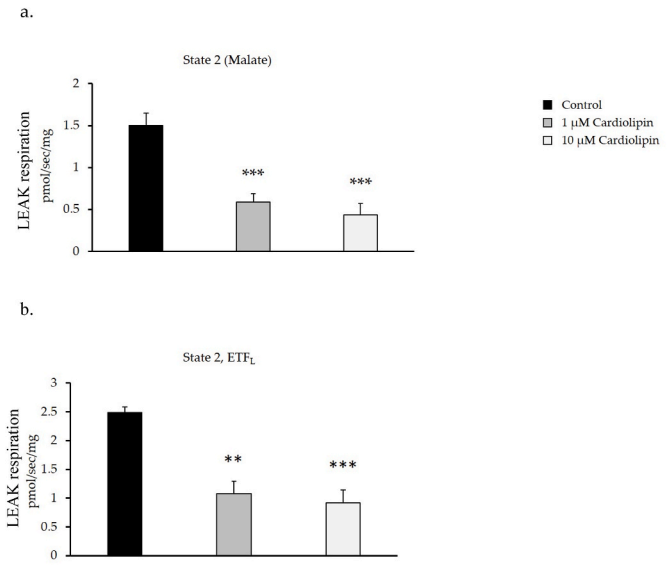

Fig. 2. Mitochondrial LEAK capacity per mg tissue measured in permeabilized muscle fibers (n = 8). Black bar represents control and the two grayscale bars represent cardiolipin 1 and 10 μM respectively. (**a**) LEAK respiration with Malate, complex I linked substrate, and (**b**) Octanoylcarnitine, lipid LEAK linked substrate (** indicates P < 0.01 and *** indicates P < 0.001, when compared with control levels).

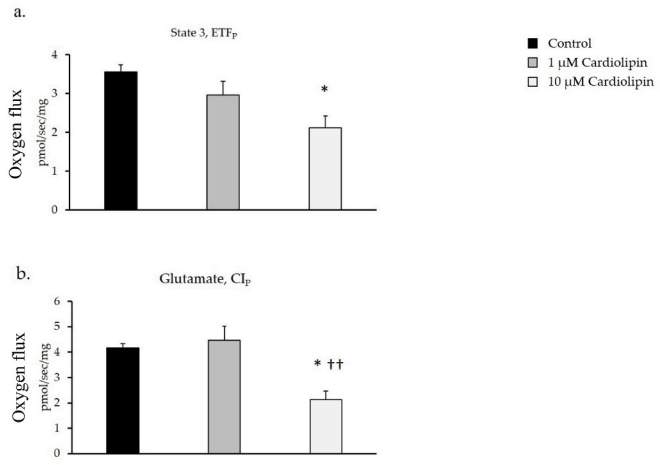

Fig. 3. Mitochondrial OXPHOS capacity per mg tissue measured in permeabilized muscle fibers (n = 8). Black bar represents control and the two grayscale bars represent cardiolipin 1 and 10 μM respectively. (**a**) ETF$_P$ after addition of ADP and (**b**) oxygen consumption after addition of glutamate in an ADP stimulated environment (* indicates P < 0.05 and †† indicates P < 0.01, when compared with control levels).

between control and 1 μM ($p < 0.01$) as well as control and 10 μM ($p < 0.01$) CL.

As seen in Fig. 5a, addition of oligomycin, a Complex V inhibitor, in presence of CL gave a significant reduction of respiration [8.16 ± 0.66,

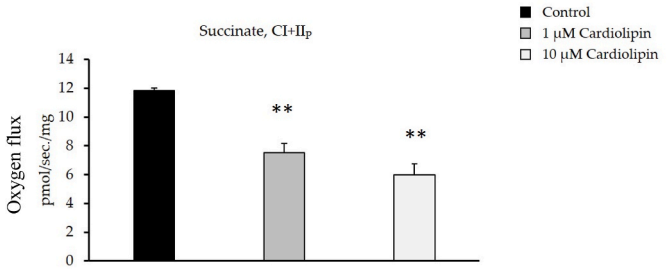

Fig. 4. OXPHOS capacity with complex I and II linked substrates after addition of succinate. (n = 8). (** indicates P < 0.01, when compared with control levels).

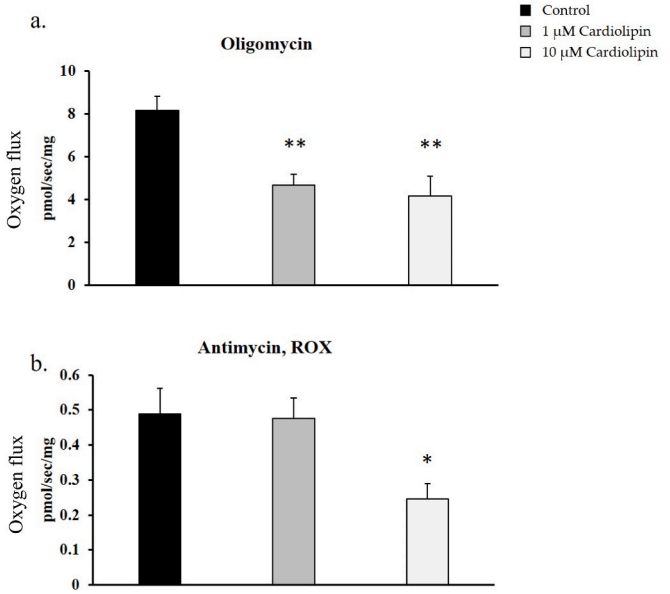

Fig. 5. **(a)** State 4 respiration induced by addition of oligomycin inhibits the F_o unit of ATP synthase and serves as an indicator of the degree of uncoupled respiration or proton leak. (**b**) Addition of antimycin A inhibits Complex III and thereby the oxidative phosphorylation. (* indicates P < 0.05 and ** indicates P < 0.01, when compared with control levels).

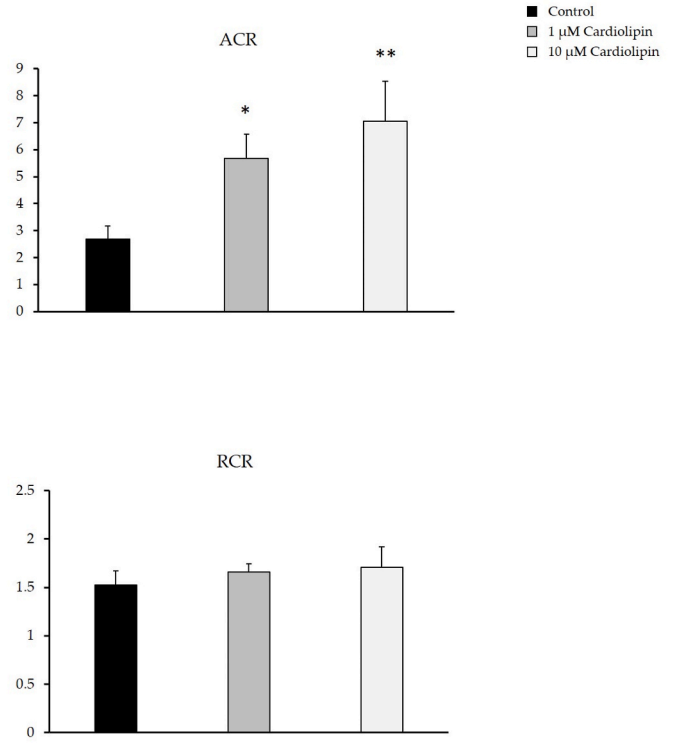

Fig. 6. (**a**) The acceptor control ratio (ACR), the relative quality of phosphorylation determined by dividing ADP by malate average rates of respiration. The data illustrate a significant increase in the ACR with increasing concentrations of cardiolipin compared to control, n = 8. This demonstrates an increase in the coupling between the oxidation and phosphorylation with an increasing concentration of cardiolipin. (**b**) The respiratory control ratio (RCR), state 3 over state 4 respiration demonstrate a non-significant effect of either cardiolipin concentration (1 and 10 μM, respectively), n = 8. (* indicates P < 0.05 and ** indicates P < 0.01, when compared with control levels).

4.67 ± 0.50 and 4.17 ± 0.91 pmol/s/mg for CON, 1 and 10 μM CL, respectively] for n = 8 [F(2, 21) F = 9.38, p = 0.001]. Fig. 5b demonstrates the addition of Antimycin A, a Complex III inhibitor which revealed no differences for 1 μM CL but a significant reduction in oxygen consumption for the 10 μM CL group [0.49 ± 0.20, 0.48 ± 0.13 and 0.25 ± 0.12 pmol/s/mg for CON, 1 and 10 μM CL, respectively] for n = 8 [F (2, 21) F = 5.17, p = 0.015].

Fig. 6a [2.69 ± 0.49, 5.69 ± 0.87 and 7.06 ± 1.49 for CON, 1 and 10 μM CL, respectively] demonstrates the effect of CL on acceptor control ration (ACR) which indicated that CL improves the coupling between oxidation and phosphorylation when comparing control and 1 (p < 0.05) and control and 10 μM (p < 0.01) CL for n = 8 [F(2, 21) = 4.63, p = 0.02]. For the respiratory control ratio (RCR) in Fig. 6b, we did not detect any significant changes induced by CL [1.52 ± 0.15, 1.66 ± 0.09 and 1.71 ± 0.22 for CON, 1 and 10 μM CL, respectively.

4. Discussion

Our major finding is that '*in situ*' mitochondrial respiration in permeabilized smooth muscle cells is attenuated when physiological concentrations of CL are applied acutely.

CL is required for optimal activity of complex I-V and participate in the structural organization and stabilization of the respiratory chain complexes (Eble et al., 1990; Robinson, 1993; Schägger, 2002). This ubiquitous and intimate association between CL and energy-transducing membranes suggest it has an important role in bioenergetics. Consequently, one would assume that CL addition would improve the mitochondrial organization and potentially increase the efficiency of electron/proton flux while minimizing the generation of toxic reactive oxygen species that correlate with cardiovascular disease. However surprisingly, in our study, when adding excess CL, we saw an acute decrease in oxidative phosphorylation using various substrates and inhibitors. As seen in Figs. 2–4, both resting (state 2) and ADP-stimulated (state 2), coupled as well as lipid respiration were significantly reduced in the CL-treated samples. Addition of succinate (Fig. 4) in an ADP-stimulated environment, which triggers a more physiological, parallel electron supply from both NADH (complex I) and $FADH_2$ (complex II), was also reduced for both 1 and 10 μM CL.

The reason for these rapid changes in oxidative phosphorylation remain unknown, but an increase in CL could function as a trap that anchors the protons essential for driving the ATPase pump (Haines and Dencher, 2002). The function of these could be to create a high buffering capacity to the membrane interface by allowing the CL to lessen the change in pH across the inner membrane. It is possible that an excess concentration of CL could bind protons and consequently temporarily limit their movement.

In our experiments, we noticed that the oxygen flux was still significantly lower in CL-treated samples upon the addition of oligomycin (Fig. 5), a well-known inhibitor of mitochondrial ATPase/ATP synthase, and that the drop was of lower relative magnitude compared

to the controls. There was no increased effect of adding more CL, suggesting that the CL concentration at 1 μM was high enough to trap the protons.

We show diminished mitochondrial respiration in the presence of CL, a phenomenon known to result in a lowering of the inner membrane potential and prevention of ROS emission Chouchani et al. (2013). Other studies have demonstrated that transient metabolic shutdown by use of mitochondrial inhibitors can be a powerful protective tool (Elrod et al., 2007; Jespersen et al., 2017). Most mitochondrial inhibitors, such as rotenone, have strong effects on the electron transport chain while being toxic for humans (Lesnefsky et al., 2004). In this regard, the added CL seems to directly affect the electron transport chain while providing a reversible inhibition of mitochondrial respiratory activity (Tatsuta and Langer, 2017). We have previously reported that oxidative phosphorylation in dedifferentiated VSMCs, when corrected for organelle density, is associated with higher ADP-restricted as well as ADP-driven respiration which suggests that increased level of cellular bioenergetics is an important factor for atherogenesis (Scheede-Bergdahl et al., 2017). Consequently, the use of reversible CL could be a future therapeutic alternative to prevent mitochondrial derived injury, especially when originating from Complex I. In addition, this is supported by the significantly higher ACR (Fig. 6a) seen after addition of CL as the higher values indicates better coupling, an increase in oxidative efficiency and consequently, a decrease in ROS production.

Several studies have demonstrated that CL interacts with, and is required for a number of mitochondrial carrier proteins (Klingenberg, 2009). For example, CL associates with pyruvate transporters, which when disrupted could affect acetyl coenzyme A and ATP derived through glycolysis (Chicco and Sparagna, 2007; Paradies et al., 2014). However, this cannot be the mechanism in our study as we were using saponin-permeabilized cells and consequently had no diffusion limitation due to carrier proteins while still observing rapid effects by CL.

Since we are demonstrating the acute effects of CL, no immunoblotting or measurements of mitochondrial density were done as the turnover rate for the corresponding mitochondrial proteins is longer than the 3-h experiment. Previous studies have demonstrated that CL associates to complexes I-V (Schägger, 2002). However, our results indicate that acute addition of physiological concentrations of CL specifically affects mitochondrial respiration in complex I. We hypothesize that this could be due to the affinity or the proximity of CL's binding to the proton channels.

Some study limitations should be acknowledged. We used octanoylcarnitine as this medium sized free fatty acid has been shown to give consistent results in rodents. However, it might not be the most common free fatty acid and it is possible that others may metabolize differently. In addition, we used chemically permeabilized smooth muscle cells for the high resolution respirometry experiments. While this is a very good method to study mitochondria *in situ*, the resulting lack of diffusion limitation through the plasma membrane could affect the true *in vivo* consequences of CL release (Pesta and Gnaiger, 2012).

Hamsten et al. (1986) observed a number of patients with elevated anti-CL antibody levels in a group of survivors following myocardial infarction, indicating that CL is released upon cardiomyocyte necrosis. However, systemic effects of this translocation has not been investigated which led us to study the acute effects of physiological concentrations of CL on VSMC mitochondria. Our findings indicate that the acute addition of CL (mimicking post-myocardial infarction events) significantly decrease the capacity to oxidize glucose and fatty acid substrates. These changes may underlie a need for the vasculature to adapt to a decreased supply of oxygen due to a reduction in cardiac ejection efficiency. More information is required to establish the mechanistic underpinnings and whether this relates to a protective response following a heart attack.

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Institutional review board statement

All procedures were approved by the Animal Ethics Committee of Concordia University (#30000259) and were conducted in accordance with guidelines of the Canadian Council on Animal Care.

CRediT authorship contribution statement

Deema Galambo: Methodology, Formal analysis, Writing – original draft. **Andreas Bergdahl:** Conceptualization, Methodology, Formal analysis, Resources, Writing – original draft.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Deema Galambo reports financial support was provided by Canadian Institutes of Health Research.

Data availability

Data will be made available on request.

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